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REMARKS

Reconsideration is requested.

Claims 1-29, 41-51 and 54-64 are pending.

The claims have been amended to obviate the Section 112, second paragraph, rejection of claims 1-29, 41-51 and 54-64. While not believed to be necessary, the claims have been amended to advance prosecution. Specifically, the applicant believes that the collection of unbound plasmid DNA from the complex in subparagraph (c) in claim 1 would be recognized by one of ordinary skill in the art as a process of collecting unbound plasmid DNA separate from the noted complex. The typographical error in claim 14 has been amended as suggested by the Examiner whose helpful comment is appreciated. Finally, claims 17, 41 and 54 have been amended to confirm that the second bound mixture contains supercoiled plasmid DNA bound to the hydrophobic interaction media.

Withdrawal of the Section 112, second paragraph, rejection of claims 1-29, 41-51 and 54-64 is requested.

As noted above, the applicant requests an interview with the Examiner and the Examiner's Supervisor in the event the present paper is not believed to place the application in condition for allowance. The interview is requested prior to the issuance of a further Action. The Examiner is requested to contact the undersigned to arrange a time convenient to the Examiner and the Examiner Supervisor for such an interview.

The Section 112, first paragraph "written description", rejection of claims 1-13, 16-29, 41-51 and 54-64 is traversed. Similarly, the Section 112, first paragraph "enablement", rejection of claims 1-29, 41-51 and 54-64, is traversed. Reconsideration

and withdrawal of the rejections are requested via the following comments as well as the attached.

The rejections are treated together in the following comments as the issues raised by the Examiner are believed to be related. Specifically, the Examiner asserts that the application allegedly fails to provide adequate written description for the recited "hydrophobic interaction media" as well as the various recited conditions to be used in the process of separation. The applicant respectfully submits however that patent applications are not required to be detailed user manuals and are rather directed to those of ordinary skill in the related art. As explained below, the terms "hydrophobic interaction media" and various separation conditions, as recited in the claims, are well recognized by those of ordinary skill in the art and were believed to be recognized by Examiner Sandals throughout the three (3) substantive Office Actions which have been issued in the present application over the last 1 ½ years. The Examiner is urged to appreciate that Examiner Sandals has previously cited a number of references as either allegedly anticipating or making obvious the presently claimed invention. Clearly, these references, which are directed to those of ordinary skill in the art and have been, in part issued U.S. patents, describe various interaction media and reaction conditions which are indicative of the advanced level of skill in the present art.

The Examiner is requested to see, for example, the claims of previously cited Kitamura (U.S. Patent No. 6,441,160), which recite "hydrophobic interaction chromatography" and further describes various elution steps as well as other process steps which allow adsorption and desorption from the reaction media such as changing conditions surrounding the media. These process steps are well known to those of

ordinary skill in the art and one of ordinary skill reading the present application would appreciate the applicant was in possession of the claimed invention at the time the application was filed. Moreover, practice of the presently claimed invention would not required undue experimentation. Consideration of the following in this regard is requested.

The Examiner will appreciate that claims specify hydrophobic interaction media, which will be appreciated by one of ordinary skill to include a hydrophobic ligand attached to a base matrix. The base matrix exemplified in the present application was in the form of either a spherical bead made with methacrylate polymer, copolymer of methacrylate ethylene glycol or a cross linked agarose backbone. The mechanism of the hydrophobic interaction is not dependent on the base matrix. There are innumerable "base matrices" that are commercially available with a variety of hydrophobic ligands attached to them. The base matrices differ in their rigidity, porosity etc, which do not determine selectivity or specificity. One ordinarily skilled in the art can screen through these matrices using very simple experiments, without significant effort or undue experimentation. A list of commonly used base matrices is provided in the attached Reference from "Protein Purification" (Protein Purification, Principles, High Resolution Methods, and Applications; Edited by Jan-Christer Janson & Lars Ryden. ISBN 0471-18626-0, 2nd Edition (1998)). A general description of hydrophobic interaction media, such as is contained in the present application, will be recognized by one of ordinary skill in the art to include a complete description of a variety of such interaction media.

Several chromatographic methods that were developed using the spherical beads as base matrices have been readily converted to perform similarly on other non-spherical matrices such as membranes, etc. For example, commercial manufacturers such as PALL, Millipore, etc. have been attached ion exchange ligands such as Quarternary Ammonium ion to membranes and have demonstrated separation of proteins with similar performance as the "bead" configuration. The differences are essentially the porosity and flow characteristics which do not inherently affect the selectivity or specificity of the interaction.

Hence, one of ordinary skill would reasonably conclude that the presently claimed invention can be readily practiced, without undue experimentation, with different spherical and non-spherical base matrices by following readily available standard procedures for attaching the ligands to such surfaces. (See Attachment 1: Comparison of chromatography based Q and Membrane based Q).

Moreover, one of ordinary skill will appreciate that elution of the bound component from a hydrophobic interaction media is typically achieved by reducing the hydrophobic interaction between the bound component and the ligand. Typically, the hydrophobic interaction is reduced, for example, by 1) changing the salt concentration 2) changing the polarity of the solvent 3) using detergents (See attached Reference, page 301). The present application exemplifies the first condition to alter the interaction between the bound molecule and the ligand. Conditions (2) and (3) were not used in the examples of the application however one of ordnary skill in the art may reasonably expect to utilize these or a combination of these available alternatives for changing hydrophobicity. The applicant notes that detergents are generally used when the

hydrophobic interaction is strong, whereas in the exemplifications of the present invention the interaction is weak.

Typically, pH changes are not used alone to displace the components as continual variation of pH alone during the elution process is generally more difficult to maintain and hence is generally avoided as a sole change in condition in manufacturing processes (See the attached Reference; page 301).

Processes are typically and practically conducted at a set temperature and temperature change is typically not used alone as a method for displacing the components bound to a hydrophobic interaction media. One of ordinary skill in the art can readily determine however the individual and/or combined effects of temperature, pH and salt concentration on the elution pattern from a relatively simple set of experiments. The applicant submits that the application describes and enables more than the exemplified embodiments of the disclosed invention. The claims are submitted to be supported by an adequate written description which teaches one of ordinary skill in the art how to make and use the claimed invention.

As for the Examiner's suggestion that the application does not provide enablement for any type of salt at any concentration or any type of hydrophobic interaction media, consideration of the following is requested.

The examples use ammonium sulfate as the salt for effecting the binding through hydrophobic interaction and the elution (see discussion above) is effected through decrease of the hydrophobic interaction through decrease of the ammonium sulfate concentration.

It is well known in the art that the hydrophobic interaction can be effected by a range of salts. The attached Reference 1, at page 287, for example, clearly describes this as follows "The influence of different anions on hydrophobic interaction follows the well known Hofmeister series. Anions that promote hydrophobic interaction most are to the left in the series. The anions to the right are called chaotropic:

Sulfate > Cloride > Bromate>Nitrate>ClO_4- >Iodide> SCN-

Also, cations influence the strength of interaction:

Magnesium > Lithium>Sodium>Potassium>Ammonium"

This series above suggests that one ordinarily skilled in the art can choose with considerable degree of predictability the salt(s) that will provide the necessary hydrophobic interaction.

Thus, the specification is submitted to teach mroe than the use of sodium chloride and ammonium sulfate as other salts can be used with equal effectiveness by simple experiments consisting of the combination of the above anions and cations at various concentrations. Moreover, the examples provide clear guidance to the relative "strength" of interaction needed, which provides one ordinarily skilled in the art with adequate information to readily substitute other salts/concentrations.

As for the reference to hydrophobic interaction media, the examples in the current application demonstrates the effectiveness of butyl and octyl chemistries (ligands) in effecting the hydrophobic interaction. The Examiner appears to believe that the current application fails to enable other ligands.

These examples by themselves provide evidence that the interaction is not specific to a base matrix (demonstrated with Agarose base matrix and methyacrylate

polymer base matrix) or to a specific ligand (demonstrated with butyl – a C4 ligand and an octyl – a C8 ligand). This suggests that one ordinarily skilled in the art is able to choose other ligands such as C5, C6, C7, etc. to effect the same hydrophobic interaction with minor modifications in the salt concentrations.

The attached Reference, pages 292-296, provides, for example, a summary of both a variety of commercially available ligands and possible chemistries, along with possible base matrices that can be readily used for hydrophobic interaction. These ligands, just like the salts, follow a certain trend in their strength of hydrophobic interaction. In general, the strength of interaction increases with the increase in length of the ligand for alkyl types of ligands. (See, attached Reference).

Moreover, the hydrophobic interaction strength is the combination of ligand strength and salt strength, each of which can be readily manipulated to achieve certain strength of interaction equivalent to that demonstrated in these examples.

In summary, the present application teaches one of ordinary skill in the art to make and use the claimed invention, as well as the exemplified embodiments. As mentioned in previous discussions, the base matrix has minimal influence on the strength of interaction and specificity and hence one ordinarily skilled in the art is able to choose an appropriate base matrix (spherical bead, membrane, etc.) to carry out the methods of the claimed invention.

The Examiner appears to suggest that the claims are broad in including the use of any type of hydrophobic media and any salt concentration. As discussed above, the nature and strength of the hydrophobic interaction can be obtained by several criteria, such as combinations of salt concentrations and ligand strengths as well as possibly

temperature and pH. One ordinarily skilled in the art, hence is not restricted to the specific ligands used in the examples to obtain the same hydrophobic interaction. The claims are therefore submitted to be supported by an enabling disclosure.

The specification teaches a method for purification of plasmid DNA, for example, using a certain strength of hydrophobic interaction between the components in the mixture and the ligand and using a different (reduced) strength of interaction to displace(elute) the bound components. The specification provides one ordinarily skilled in the art with an indication or example of the relative strength of interaction needed to bind and elute the components of the mixture, by exemplifying conditions of salt concentration, ligand, etc. One ordinarily skilled in the art, with this information is able to choose from available hydrophobic interaction media, ligands and, for example, salt concentrations that will provide similar strengths of interaction as exemplified. This can be accomplished through simple experiments by one ordinarily skilled in the art, as there is ample literature information to determine approximate strengths of interaction.

While the art available at the time the present application was filed may not teach a method for purification of plasmid DNA using hydrophobic interaction media, there is an abundant amount of literature information that clearly describes hydrophobic interaction phenomena and related methods to carry out the simple experiments to make and use the current invention. Specifically, hydrophobic interaction media is commercially available from several established vendors with ligands with different strengths. Extensive literature exists on establishing the strength of ligands and, for example, strength of salts. Since hydrophobic interaction has been used with other

macromolecules extensively, there is a significant knowledgebase for one ordinarily skilled in the art to readily use the current invention without undue experimentation.

Withdrawal of the Section 112, first paragraph, rejections noted above is requested.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. Alternatively, as requested above, the Examiner is requested to contact the undersigned to arrange an interview prior to issuance of a further Action.

Respectfully submitted,

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Attachment 1

Performance Characteristics	Intercept Q Membrane Adsorber	Column Technology
Mass Transport	Surface capture	Diffusion limited
Separation Time	***FAST*** • Low residence times (< 1 sec) with highly efficient separations – linear velocities up to 1,000 cm/hr • High membrane permeability – typical linear velocity 350 cm/hr	Standard chromatographic residence time are measured in minutes, limited by diffusion
Flow Distribution	***CONSISTENT*** Even flow allows full-utilization of charged sites over all membrane	Dependent upon column packing – channeling causes premature breakthrough
Throughput Speed	***HIGH PERFORMANCE*** • High throughput (g/hr) • High linear velocities: Minimal mass transfer/kinetic limitations • Insensitive to flow rate	Pressure limited requiring oversized columns
Physical Characteristics		
Low Bed Heights	***EFFICIENT*** Intercept Q devices are ~100x more efficient	Column packing, high bed heights
Low Bed Volumes	Ultra-low membrane bed volume required to adsorb trace impurity load	Limited by pressure drop and packing
Hold-up Volume	Minimal	Column dependent
Size	Lightweight plastic capsules	Large footprint
Set-up	***EASY TO USE*** • Plug in and process operation • No re-use validation • Single-use, disposable design • Prestacked membrane • Integrity testable	Requires: • Sanitization • Large volume buffer, preparation steps • Column packing • Resin storage

PROTEIN PURIFICATI

Principles, High-Resolution Methods, and Applications

SECOND EDITION

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CONTENTS

7.1 INTRODUCTION

Hydrophobic molecules in an aqueous solvent will self-associate. This association is due to hydrophobic interaction. The hydrophobic interaction is of prime importance in biological systems. It is a major driving force behind the folding of globular proteins (e.g., formation of the molten globule state), in the association of protein subunits, and in the binding of many small molecules to proteins as in enzyme catalysis, regulation, and transport across surfaces. It is also responsible for the self-association of phospholipids and other lipids to form the biological membrane bilayer and the binding of integral membrane proteins.

In hydrophobic interaction chromatography (HIC) the hydrophobic interaction is utilized for the binding of proteins to adsorbents with hydrophobic ligands. Our present, rather detailed, knowledge of the protein three-dimensional structure has revealed that the surface of globular proteins can have extensive hydrophobic patches in addition to the expected hydrophilic groups. It is these hydrophobic regions that bind to hydrophobic ligands on the adsorbent, in media favoring hydrophobic interaction (e.g., an aqueous solution with a high salt concentration). Elution (and separation), according to differences in the strength of interaction between the proteins and the amphiphilic matrix, is generally brought about by decreasing the salt concentration of the eluent. In some cases a decrease of the solvent polarity is also needed.

The concept of separation proteins under HIC conditions was outlined by Tiselius already in 1948,[1] but the technique has been developed since the early 1970s. The first gels of practical use for HIC were of a mixed hydrophobic–ionic character.[2–4] Neutral adsorbents (alkyl and aryl ethers) were later prepared by Porath and co-workers[5] and Hjertén and co-workers,[6] the latter leading to the introduction of octyl- and phenyl-Sepharose. It was also Hjerte'n who suggested the now generally accepted name of the technique: hydrophobic interaction chromatography.[7] The term hydrophobic chromatography should be avoided, as it is the interaction between the solute and the adsorbent that is hydrophobic, not the chromatographic procedure.[8] The HIC technique has been adapted to the HPLC mode using the traditional gel material agarose,[9] as well as organic polymers[10] and silica-based matrices.[11] Alternative methods for immobilization of hydrophobic ligands, (e.g., attachment of alkyl sulfids to oxirane-activated agarose[12]) have been developed.

Salting-out chromatography and other types of chromatography related t HIC will be discussed briefly, as will other types of chromatographic adsorbent developed more recently.[13]

Adsorbents for reversed-phase chromatography (RPC) and HIC both con tain hydrophobic ligands. In the RPC adsorbents the ligand density is muc higher than in those used for HIC. Although the separation of both types c adsorbents is based on hydrophobic interaction, the mechanism on the molecu lar level is different. Whereas an RPC adsorbent can be regarded as a continu ous hydrophobic phase, the ligands on a HIC adsorbent interact individuall with the solutes. As a result, globular proteins very often denature whe applied on RPC columns, which are therefore used mainly for peptides an small proteins. RPC also requires more drastic conditions for elution, such a a gradient of organic solvents, as compared to HIC. HIC thus has a mor general field of application. RPC is described in Chapter 6.

In order to give the reader a general understanding of the principles o hydrophobic interaction chromatography, this chapter discusses the techniqu from both a theoretical and a practical point of view. Finally, some application of the HIC technique will be described.

7.2 THE HYDROPHOBIC INTERACTION

7.2.1 Theory

Hydrophobic interactions in aqueous solvents are considered to be drive primarily by interactions within the solvent and to a lesser extent by interac tions between the nonpolar solutes. However, in the case of protein folding it has also been shown that van der Waals interactions between nonpola amino acid side chains are significant.[14] It has also been shown that van de Waals interactions also play a role in chromatographic separations.[15] But t what degree van der Waals interactions influence the binding between protein and ligands on a HIC gel is not known, and will thus not be discussed furthe in this chapter. For a detailed treatment of hydrophobic interaction, see Refs 16–18. A short theoretical discussion follows, as a background to the HIC tech nique.

Water is a poor solvent for nonpolar solutes. Dissolving a nonpolar sub stance in water is thermodynamically unfavorable. Because of its hydroge bonding capability, water has a unique structure, causing a high surface tension A nonpolar solute forces the water to form a cavity in which the solut fits (Figure 7-1A). In the process, many hydrogen bonds between the wate molecules are broken, but new hydrogen bonds are formed among the wate molecules surrounding the cavity, leading to a negative change in enthalpy ΔH. This change is due only to a small extent to the weak van der Waal interaction between the solute and the surrounding water. The increase i

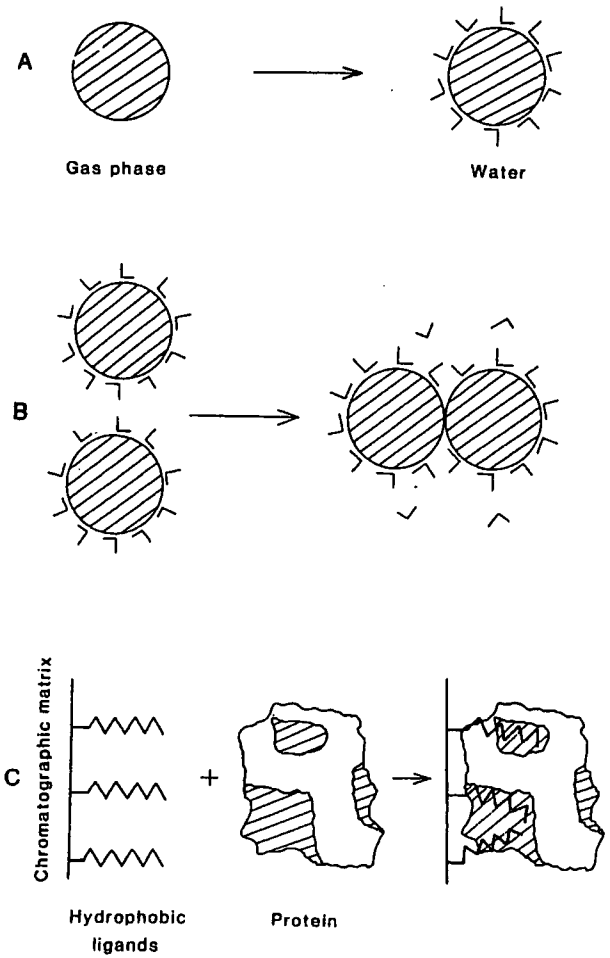


FIGURE 7-1. Schematic representation of the hydrophobic interaction. (A) Dissolving of a nonpolar solute in water. (B) The interaction of two nonpolar solutes in water. (C) A hypothetical representation of an interaction of ligands of an amphiphilic gel and hydrophobic surfaces on a protein. L represents water molecules.

the degree of order of solvent molecules near the solute explains the negati change in entropy, ΔS, that results.

The well-known expression

$$\Delta G = \Delta H - T\Delta S \quad (7-$$

describes the change in free energy, ΔG, of a process. A negative value ΔG implies that the process is thermodynamically possible. The dissolutic of a solute is thus favored either by a negative change in enthalpy or by positive value of the entropy change. For the cavity-forming process earlie the net change in free energy is positive, which makes it unfavorable.

In hydrophobic interaction (Figure 7-1B), the increase in entropy (ΔS 0) originating from water molecules leaving the more ordered structure arour the nonassociated solutes for the more unstructured bulk water is the ma driving force. The net decrease in cavity surface area that occurs when tv nonpolar solutes associate explains why some water molecules go over to tl bulk phase. The positive enthalpy term, ΔH, is smaller than the entropy ter and does not influence the spontaneous association to a great extent.[19]

The interaction between an amphiphilic matrix and hydrophobic areas (a protein (Figure 7.1C) can be explained in the same way as the interactic between nonpolar solutes in water (Figure 7-1B).

Both entropy and enthalpy change with temperature for hydrophobic inte actions. A theoretical treatment of the temperature dependence of the hydr phobic interaction is thus complicated, but one can conclude that the streng of hydrophobic interactions should increase with an increase in temperatur at least in the temperature range that is of interest for HIC.

Two factors of great importance for HIC are the type and concentratic of salt used and additives that change the polarity of the solvent. The latte exemplified by ethylene glycol, decrease the interaction between the HIC g and proteins by changing the overall structure of water slightly toward structure resembling an organic solvent.

The influence of different anions on hydrophobic interaction[20] follows tl well-known Hofmeister (lyotropic) series.[21] Anions that promote hydrophob interaction most are to the left in the series. The anions to the right—ClO_4 I^-, and SCN^-—are called chaotropic:

$$SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^- \quad (7-$$

Also, cations influence the strength of interaction:

$$Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+ \quad (7-$$

Melander and Horvath[22] have shown that the effectiveness of differe salts in promoting hydrophobic interactions can be explained by their contrib tion to the surface tension of the solution. The formation of a cavity in wat with a salt that gives a high surface tension needs a bigger input of ener

containing a salt giving lower surface tension. The higher the salt concentration, the stronger the interaction. It is the salts that increase the surface tension most that give the strongest hydrophobic interaction.

Factors other than surface tension can also affect the hydrophobic interaction. Protein hydration and specific interactions between the protein and the salt ions seems to be factors that influence the strength of interaction.[23]

7.2.2 The Hydrophobicity of Amino Acids and Proteins

Although most hydrophobic amino acids are buried in the interior of globular proteins, and hydrophilic amino acids have a tendency to be exposed on the surface, some hydrophobic amino acids also appear on the surface. For globular proteins it has been shown that only about 20% of the amino acid side chains are totally buried.[24] The hydrophobicity of protein surfaces is the sum of the hydrophobicities of the exposed amino acids and part of the backbone. A discussion of protein surface hydrophobicity is thus based on the hydrophobicity of amino acids.

Two approaches have been used for the estimation of the hydrophobicity of amino acids. The first is based on direct measurements of the solubilities of individual amino acids in water and organic solvents.[25-28] A hydrophobicity scale for the different amino acids has thus been constructed on the basis of free energy of transfer for the amino acids from ethanol or dioxane to water.

The second approach is based on the empirical inspection of known protein structures.[29-33] Here, several hydrophobicity scales are based on, for example, the environment of the different amino acids, the fraction of amino acids that is buried in the protein, a side chain interaction parameter, or a fractional accessibility to the surrounding solvent of the different residues. For a comparative study of hydrophobicity scales published, see Ref. 34.

Table 7-1 is a comparison of two scales of hydrophobicity, the first based on amino acid solubility[25-28] and the second based on the fraction of amino acids buried within proteins[30] (average values obtained from 20 proteins with known structures). Some differences are striking. Proline is a rather hydrophobic amino acid, but its secondary structure-breaking properties make it appear in bends typically on the surface of proteins. Lysine is also classified as a rather hydrophobic residue in the scales based on solubility studies, although it is the most exposed of the amino acids. The four methylene groups of the lysine side chain disfavor the solubility of this amino acid in water, whereas the amino group with its hydrogen-binding capability has a strong tendency to be exposed on the surface of proteins.

To get more accurate values for the hydrophobicities of amphiphilic amino acids, one report suggests a scale based on the hydrophobicity of each individual atom.[35]

A linear relationship exists between the logarithm of the solubilities of hydrocarbons and the surface area they form in water.[36] The cavity area is the same as the accessible surface area of the hydrocarbons.

Protein surfaces are not smooth, but are rather rough and complex. Analyses of protein surfaces often use the method of Lee and Richards.[37,38] Figure

TABLE 7-1. Hydrophobicity Scales for Amino Acids

Scale I (kcal/mol)		Scale II (Fraction Buried)	
Trp	3.77	Phe	0.8
Ile	3.15	Trp	0.8
Phe	2.87	Cys	0.8
Pro	2.77	Ile	0.7
Tyr	2.67	Leu	0.7
Leu	2.17	Met	0.7
Val	1.87	Val	0.7
Met	1.67	His	0.7
Lys	1.64	Tyr	0.6
Cys	1.52	Ala	0.5
Ala	0.87	Ser	0.4
His	0.87	Arg	0.4
Arg	0.85	Asn	0.4
Glu	0.67	Gly	0.4
Asp	0.66	Thr	0.3
Gly	0.10	Glu	0.3
Asn	0.09	Asp	0.3
Ser	0.07	Pro	0.3
Thr	0.07	Gln	0.3
Gln	0.00	Lys	0.3

Scale I is based on the solubility of the different amino acids, expressed as free energy of transf for the amino acids from ethanol or dioxane to water.[25-28] Scale II is based on the fraction the number of the different amino acids buried within proteins[30] (average values obtained fro 20 proteins with known structures).

The accessible surface (see legend to Figure 7-2), with a water molecu as the probe, is used when protein surfaces are discussed. The nonpolar surfa is defined as the area containing side chains with carbon and sulfur and mai chain carbon atoms (hydrogen atoms are not considered). The proportion nonpolar surface area does not differ much among the globular protei examined. A report on the subject gives figures of 41% for lysozyme, 48% f myoglobin, and 46% for ribonuclease.[37]

In one report,[39] 46 monomeric globular proteins with known structur were studied, and their surfaces and interior examined. This report also fou that the proportion of hydrophobic surface area does not differ much amo the proteins. With the definition of nonpolar compounds used in this repo the nonpolar fraction of the surfaces varied between 50 and 68%.

7.2.3 Interaction between Protein and the Hydrophobic Interaction Chromatography Adsorbent

No general and definite answer can be given to the question of how a prote surface should look to be able to interact with hydrophobic ligands on a H

native protein and consisted of several more or less unfolded species of α-lactalbumin. A rerun of the second peak again gave two peaks, showing that the unfolding is reversible. The higher the temperature, the larger the proportion of the α-lactalbumin that elutes with a partly unfolded structure.

The retention also increases with temperature. This is also the case for the more stable lysozyme,[46] but to a lesser extent. One concludes that the temperature effect in HIC is due to two factors: (1) the increase in hydrophobic interaction with temperature and (2) a temperature-dependent alteration of the structures of proteins, especially labile ones.

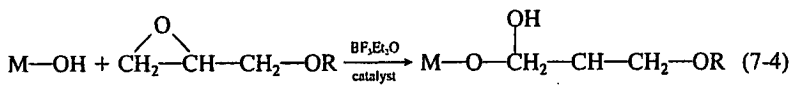
7.3 ADSORBENTS FOR HYDROPHOBIC INTERACTION CHROMATOGRAPHY AND ADSORBENTS RELATED TO HYDROPHOBIC INTERACTION CHROMATOGRAPHY

7.3.1 Adsorbents for Hydrophobic Interaction Chromatography

Many types of matrices are suitable for preparing adsorbents for HIC, but the most extensively used has been agarose. When the technique has been adopted to HPLC, silica and organic polymer resins have also been employed.

A number of commercially available HIC adsorbents are shown in Table 7-2 and some structures of HIC adsorbents are illustrated in Figure 7-4. All structures shown (except C) are octyl matrices, but any chain length can be chosen if one prepares the matrices oneself. The first (Figure 7-4A) was introduced by Shaltiel[3] and is based on cyanogen bromide activation[47] of an agarose gel, after which coupling with an alkylamine is performed. This coupling procedure creates ligands with a positive net charge. The structure in Figure 7-4A is the isourea derivative proposed by Kohn and Wilcheck.[48]

Figure 7-4 shows the B and C types based on the glycidyl ether (with an epoxide, oxirane, functional group) coupling procedure[6] (also used for the production of Octyl and Pheny Sepharose). This coupling method is widely used, so it will be described in some detail. Some glycidyl ethers are commercially available but they can also be prepared according to Ulbrich and coworkers.[49] The reaction scheme for the coupling of the ligand to the gel (usually agarose) is as follows:



R is an alkyl or aryl group, and M is the matrix. The gel should be transferred to an organic solvent, as dioxane. This is done in steps (100-ml portions to 100-ml sedimented gel), on a Büchner funnel:

1. one washing with water–dioxane (4:1)
2. one washing with water–dioxane (3:2)
3. one washing with water–dioxane (2:3)
4. one washing with water–dioxane (1:4)
5. seven washings with dioxane

Transfer the gel to a reaction vessel equipped with a stirrer.

Add 100 ml dioxane to 100 ml of sedimented gel, and add 2 ml of a 48 solution of boron trifluoride etherate in diethyl ether. Stir for 5 min. Ac 1 ml of glycidyl ether dissolved in 10 ml dioxane dropwise from a separato funnel. The reaction takes about 40 min. After the reaction, transfer the g back to water using the previous scheme, but in reverse order, and final wash with water. The amount of ligand coupled to the gel can be controll by varying the amount of the glycidyl ether added. The glycidyl ether couplir method produces gels that are charge free, and thus should have no oth interaction with proteins than hydrophobic interaction. However, the phen group shown in Figure 7-4C, (and other aromatic groups) also has a potenti for π–π interaction.

A recently introduced coupling method that leads to the structure shov in Figure 7-4D was used for coupling hydrophobic ligands to agarose ge used as an HPLC packing.[9,50] The agarose is first activated with γ-glycidoxypr pyltrimethoxy silane in water. The immobilization of the ligands is then pe formed in the alcohol that is to be coupled to the gel. The resulting gel noncharged and also contains a spacer, making the ligands more available f the proteins.

The ligand structure in Figure 7-4E was introduced by Maisano and c workers.[12] The agarose is first activated with a bisepoxide, 1,4-butanedi diglycidyl ether, and is then coupled with an alkyl mercaptan. The gel, al charge free, contains a spacer arm. The ligand density can be regulated easi by varying the amount of alkyl mercaptan.

Polymeric coatings[51] are of great importance as stationary phases for HI Both silica and polymeric matrices can be derivatized this way. One drawba with polymeric coatings has been their slow mass transfer properties.[52,53] B modern polymeric phases have film thicknesses in the range of monolayer t to 5 nm,[54] so the difference in mass transfer velocity between polymeric a monomeric phases is small. Common polymeric phases used for HIC matric are oligoethyleneglycol and polyether.

7.3.2 Ligand Density and Capacity of Hydrophobic Interaction Chromatography Adsorbents

The density of ligands is important for the strength of the interaction betwe the adsorbent and the proteins, as well as for the capacity. For the commercial available Octyl and Phenyl Sepharose gels, the ligand density is approximate 40 μmol/ml gel bed, corresponding to a degree of substitution of approximate 0.2 mol hydrophobic substituent per mole galactose.[55] The capacity of Phen

TABLE 7-2. Commercially Available Adsorbents and Columns for HIC (the list may not be complete)

Supplier	Product Name	Functional Group(s)	Remarks
A. Conventional packings			
J.T. Baker	HI-Propyl	Propyl	Silica, 15 and 40 μm, 300 and 275 Å
Bio-Rad	Methyl HIC	Methyl	Methacrylate, 50 μm
	t-Butyl HIC	Butyl	
Pharmacia	Phenyl Sepharose	Phenyl	Agarose, 34 μm
	Butyl Sepharose 4B	Butyl	Agarose, 45–165 μm
	Octyl Sepharose CL-4B	Octyl	Agarose, 45–165 μm
	Phenyl Sepharose CL-4B	Phenyl	Agarose, 45–165 μm
	Butyl Sepharose 4 FF	Butyl	Agarose, 45–165 μm
	Phenyl Sepharose 6 FF	Phenyl	Agarose, 45–165 μm
	HiLoad Phenyl Sepharose	Phenyl	Agarose, 34 μm, prepacked columns
Sigma		ω-amino C-2, C-3, C-4, C-5, C-6, C-8, C-10, and C-12. 4-Phenylbutylamine, 2,2-diphenylpropylamine. Alkyl: C-2, C-3, C-4, C-5, C-6, C-8, C-10, and C-12. Phenyl and trityl.	Agarose 4% CL or non-CL
SynChrom	SynChroprep	Propyl	Silica, 15 and 30 μm, 300 Å
TOSOHAAS	Ether-5PW	Oligoethyleneglycol	Polymer, 20–40 μm
	Phenyl-5PW	Phenyl	Polymer, 20–40 μm
	Ether 650	Oligoethyleneglycol	Polymer, 20–50 μm
	Butyl 650	Butyl	Polymer, 40–90 μm
	Phenyl 650	Phenyl	Polymer, 50–150 μm
B. HPLC packings			
J.T. Baker	HI-Propyl	Propyl	Silica, 5 and 15 μm, 300 Å
Biochrom Labs	Hydrocell C3 1000	Allyl	Polymer, porous
	Hydrocell C4 1000	Butyl	Polymer, porous
	Hydrocell C3 NP10	Allyl	Polymer, nonporous
	Hydrocell C4 NP10	Butyl	Polymer, nonporous
Bio-Rad	Bio-Gel MP-7 HIC	Methyl	Polymer, 7 μm, 900 Å
	Bio-Gel Phenyl-5PW	Phenyl	Polymer, 10 μm, 1,000 Å
Beckman	Spherogel-HIC		Silica, 5 μm, 300
Interaction	MCI GEL CQH3xs	Butyl, phenyl	Polymer, 10 μm
	Hydrophase HP-Butyl	Butyl	Polymer, 10 μm
Mitsubishi Kasei	MCI GEL HIC	Ether, butyl, phenyl	Polymer, 10 μm, wide pore
Pharmacia	Phenyl Superose	Phenyl	Agarose, 13 μm
	Alkyl Superose	Neophentyl	Agarose, 13 μm
Showa Denko	Shodex HIC	Phenyl	Polyhydroxymethyl acrylate, wide pore
Sigma	SigmaChrom HIC-phenyl	Phenyl	Polysaccharide CL, 12–15 μm
Supelco	Supelcosil LC-HINT	Diol	Silica, 5 μm, 100 Å
SynChrom	SynChropak	Propyl, hydroxypropyl, and pentyl	Silica, 6 μm, 300 Å
TOSOHAAS	Ether 5PW	Oligoethyleneglycol	Polymer, 10, 13, and 20 μm, 1,000 Å
	Phenyl 5PW	Phenyl	Polymer, 10, 13, and 20 μm, 1,000 Å
	Butyl-NPR	Butyl	2.5 μm, nonporous
YMC	YMC-Pack-HIC	CL polyamide-containing propyl ligands	Silica, 6.5 μm, 300 Å
C. Novel separation media			
Millipore	MemSep chromatography cartridges		Continuous network of regenerated cellulose, derivatized, 1.2-μm flowthrough pores
Perseptive Biosystems	POROS PH	Phenyl	PS/DVB beads, covered by a CL polyhydroxylated polymer, large transecting throughpores
	POROS PE	Phenyl ether	
	POROS BU	Butyl	
	POROS ET	Ether	

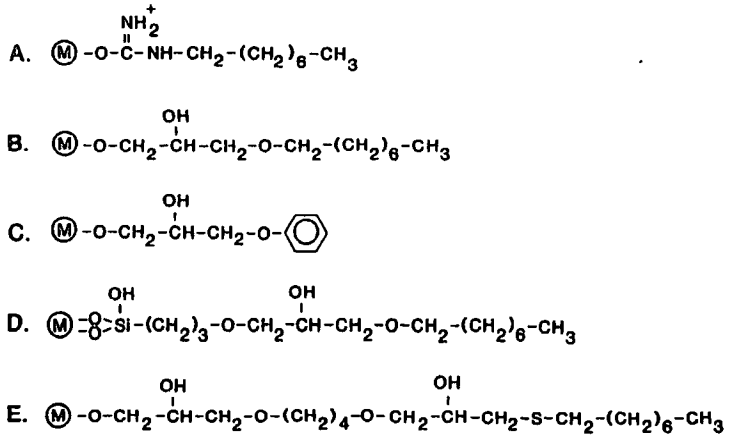


FIGURE 7-4. Different hydrophobic ligands coupled to a gel matrix, M.

and Octyl Sepharose CL-4B in 0.01 *M* sodium phosphate buffer, pH 6.8, containing 1 *M* ammonium sulfate, is approximately 15 to 20 mg human serum albumin or 3 to 5 mg β-lactoglobulin per milliliter of gel.[55] Several procedures for determining ligand density have been employed,[56,57] including NMR, gas chromatography, and elementary carbon analyses. Ligand density determination for HIC adsorbents containing aromatic groups (e.g., phenyl) can also be made by derivative ultraviolet spectroscopy,[58] and adsorbents of the type shown in Figure 7-4E can be analyzed by sulfur determination.[12]

7.3.3 Other Types of Adsorbents Related to Hydrophobic Interaction Chromatography

At high salt concentrations, matrices used for gel filtration show adsorption of proteins.[59–62] The interaction, which is of a hydrophobic nature, can be used for the separation of proteins. Matrices such as cellulose, dextran, agarose, organic polymers, and modified silica can be utilized. The technique is called salting-out chromatography.

Spacers used in affinity chromatography are often of hydrophobic nature and can in some cases make a considerable contribution to the interaction between solutes and adsorbents.[63]

In addition to the hydrophobic interaction, HIC adsorbents with aromatic ligands also show the so-called π–π interaction. This effect is explicitly utilized in the charge-transfer chromatographic technique,[64] using different kinds of ligands.

Another method related to HIC is the so-called thiophilic adsorption technique.[65,66] The ligand is divinylsulfone substituted with, for example, mercapto-

ethanol. Although the technique is related to HIC, the adsorption characteristics of the thiophilic adsorbents are different than those of HIC ones.

The binding of proteins to dye–ligand substituted adsorbents[67,68] is du partly (although far from predominantly) to the hydrophobic interaction. Alsc binding to other ligands utilized in affinity chromatography can be partly c hydrophobic nature.

7.4 CHROMATOGRAPHIC TECHNIQUES

7.4.1 Introduction

Most proteins bind to HIC adsorbents, and the technique can thus be use in many purification procedures. HIC is based on a different separation princ ple from most other separation techniques and can thus, in combination wit these, afford a high degree of purification. The mass recoveries of proteir are mostly excellent, as is the recovery of enzyme activity.

The high capacity of HIC adsorbents makes them suitable for use at a early stage in a purification scheme.[44] The similarities to salt precipitatio also based on protein surface hydrophobicity, make a combination of thes two methods less useful. However, after an initial precipitation step to remov the most hydrophobic proteins, the usual second precipitation can be replace by an HIC purification step, especially because HIC gives sharper separatio than does salt precipitation.

Because of the rather high salt concentrations of the eluate from the HI column, a subsequent ion-exchange chromatography step is not possible witl out an intermediate desalting step. The reverse is easier to perform. A HI step can, of course, be followed directly by gel filtration, whereby the salt an other additives are automatically removed.

HIC adsorbents can also be used for batch procedures, making them sui able for purification of proteins on a large scale.[44] Rigid adsorbents allowin high flow rates can be used for large-scale chromatography, as can new type of chromatography media (e.g., membrane-based and perfusion material: (Section 7.4.4)

7.4.2 Choice of Adsorbents

Adsorbents used for HIC should preferably be charge free. At low ion: strengths, the protein can interact with the positively charged amino group on HIC gels with the structure shown in Figure 7-4A. In some cases, this ca lead to an irreversible binding of the protein to the gel.[7,69]

The strength of the interaction between a protein and hydrophobic ligand on a HIC adsorbent increases with the increase in length of the ligand[56] (alk types of ligands). Ligands containing between 4 and 10 carbon atoms a suitable for most separation problems. For proteins with poor solubility i

buffers of high salt concentration (e.g., membrane proteins) HIC adsorbents with rather long ligands are recommended.[8] A phenyl group has about the same hydrophobicity as a pentyl group, although it can have a quite different selectivity compared to pentyl ligands due to the possible $\pi-\pi$ interaction. Aromatic groups on protein surfaces can interact specifically with the aromatic ligands. The oligoethyleneglycol phases are intermediate in hydrophobicity, between butyl and phenyl.

7.4.3 HPLC-HIC

The adaption of HIC, as well as other classical protein purification techniques, to the high-performance mode has been made possible by the production of beads of small and uniform size. The smaller the bead size, the better and faster the separations can be done. This was already realized by Martin and Synge in 1941.[70] Smaller bead sizes limit the number of matrices that can be utilized successfully, as smaller beads have a higher flow resistance that leads to higher pressures. Only rigid matrices can be used as high-performance packings. High-performance HIC packings have been based on silica[11] and organic polymers[10] as well as on the traditional gel material agarose.[9,71] The capability of HPLC-HIC is illustrated in Figures 7-5 and 7-6, which show separations of model proteins and serum proteins on a pentyl-agarose column.

On columns packed with nonporous materials, separations can be achieved in 2 to 3 min.[72–74] The nonporous HIC matrices are of great value for quality

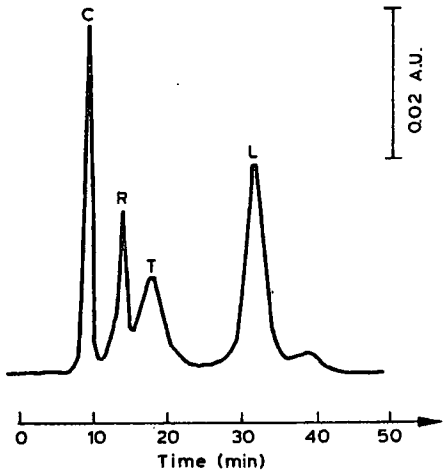


FIGURE 7-5. Isocratic separation of model proteins on a pentyl-agarose column in [illegible] (Reprinted from Ref. 9 with permission.)

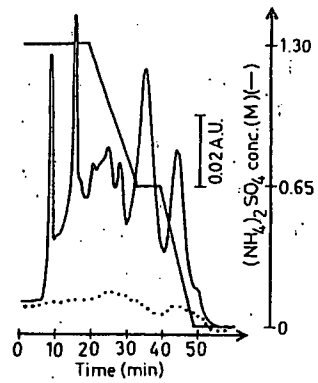


FIGURE 7-6. High-performance HIC of human serum on a pentyl-agarose gel. Elution with a negative salt gradient. (Reprinted from Ref. 9 with permission.)

control, process monitoring, and other applications that need rapid scanning of complex samples.

7.4.4 Novel Separation Media

In conventional chromatography, proteins (and other solutes) move to the outer surface of the beads by rapid convective flow through the column bed. Transport of the solutes to the inner surface (within the pores) occurs by diffusion. This process is slow, especially for conventional packing materials. By utilizing nonporous packing materials, this shortcoming is overcome, but at the cost of losing capacity. Nonporous packing materials are thus restricted to analytical applications. Some new approaches have been taken to overcome this problem. One such approach is to utilize a unified bed of through pores made from regenerated cellulose[75] or from (polymerized) derivatives of acrylamide.[76,77] A second approach is to produce particles with large enough transecting pores to allow a convective flow through the particle itself.[78,79] These materials have been utilized for different types of protein separations and are now available as HIC supports (Table 7-2).

7.4.5 Conditions for Adsorption

Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration, but due to differences in the strength of interaction between the adsorbent and different proteins, the concentration of salt needed for adsorption can vary considerably. The concentration of salt used for adsorption should be below the concentration that precipitates different proteins in the sample that should be chromatographed. For HIC gels with a ligand density similar to

Phenyl and Octyl Sepharose, the salt concentration is usually between 0.75 and 2 *M* (100% saturation is 4.05 *M*) with ammonium sulfate or 1 and 4 *M* with sodium chloride. For the most hydrophobic proteins the salt concentration can be lower. The salt is usually dissolved in a buffer solution with a concentration of 0.01 to 0.05 *M*.

Different salts give rise to differences in the strength of interaction between proteins and the HIC adsorbent. The strength of interaction as well as the capacity follows the series[20]:

$$Na_2SO_4 > NaCl > (NH_4)_2SO_4 > NH_4Cl > NaBr > NaSCN \quad (7\text{-}5)$$

Na_2SO_4, NaCl, and especially $(NH_4)_2SO_4$ are the most utilized salts in HIC. Besides the differences in the strength of interaction, the different salts also show some additional selectivity.[9]

The pH of the buffers used in HIC experiments has a decisive influence on the adsorption of proteins to the adsorbent[9] (Figure 7-7). Some proteins with high pI values bind strongly to HIC gels at elevated pH values, although

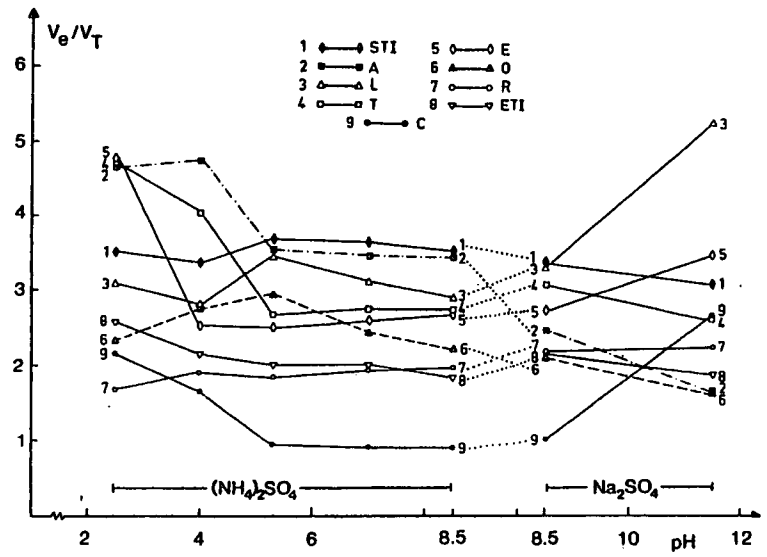


FIGURE 7-7. The pH dependence of the interaction between proteins and an octyl-agarose gel expressed as V_e/V_T (V_e is the elution volume of the different proteins and V_T is the elution volume of a nonretarded solute). Elution is by a negative linear gradient of salt. The model proteins used were STI, soy trypsin inhibitor; A, human serum albumin; L, lysozyme; T, transferrin; E, enolase; O, ovalbumin, R, ribonuclease; ETI, egg trypsin inhibitor; and C, cytochrome c. (Adapted from Ref. 9.)

no general trend in the interaction strength and protein pI values has been observed. Because the change in retardation with pH is large for most proteins, it can be worthwhile to test different pH values for adsorption. The only limitation is the stability of the protein to be purified and the stability of the chromatographic matrix (e.g., silica is not stable at high pH).

As discussed earlier, the temperature dependence of HIC is not simple, although, generally, a decrease in temperature decreases the interaction. Labile proteins should be chromatographed at low temperatures.

It is important that the column is equilibrated with the same buffer as the sample. If the sample is opalescent, it should be centrifuged or filtered before application to the column. The sample volume can be large because the proteins generally become bound to the column before elution starts.

7.4.6 Elution

Elution, whether done stepwise or with a gradient, can generally be achieved in three different ways:

1. Changing the salt concentration: The elution of solutes, in the order of increasing hydrophobicity, is accomplished by decreasing the salt concentration.
2. Changing the polarity of the solvent: A decrease in the interaction is achieved by adding solvents such as ethylene glycol or (iso)propanol. Up to 80% ethylene glycol may be used, whereas propanol should be used at lower concentrations. The addition of polarity-decreasing agents can be made after the salt has been removed from the column or concomitantly with the decrease of salt concentration.
3. Adding detergents: Detergents work as displacers of the proteins. They have been used mainly for the purification of membrane proteins by HIC.

In theory, the elution of proteins from HIC adsorbents can be accomplished by changing the salt species to a chaotropic ion (e.g., SCN^-), but this should be avoided due to the protein structure breaking properties of these ions. A change in pH may also be used for elution, but it is (so far) impossible to predict how the strength of interaction between a protein and HIC adsorbent will be affected upon changing the pH.

7.4.7 Regeneration of Hydrophobic Interaction Chromatography Adsorbents

HIC adsorbents have a long lifetime. For the regeneration, and storage, of the different HIC adsorbents available, the recommendations from the various manufacturers should be followed. For Sepharose based packings, it is recommended that the adsorbents be washed with 6 *M* guanidine hydrochloride